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Transcriptomic and Phenotypic Analyses Identify Coregulated, Overlapping Regulons among PrfA, CtsR, HrcA, and the Alternative Sigma Factors σ^B, σ^C, σ^H, and σ^L in *Listeria monocytogenes*▽†

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A set of seven *Listeria monocytogenes* 10403S mutant strains, each bearing an in-frame null mutation in a gene encoding a key regulatory protein, was used to characterize transcriptional networks in *L. monocytogenes*; the seven regulatory proteins addressed include all four *L. monocytogenes* alternative sigma factors (σ^B, σ^C, σ^H, and σ^L), the virulence gene regulator PrfA, and the heat shock-related negative regulators CtsR and HrcA. Whole-genome microarray analyses, used to identify regulons for each of these 7 transcriptional regulators, showed considerable overlap among regulons. Among 188 genes controlled by more than one regulator, 176 were coregulated by σ^B, including 92 genes regulated by both σ^B and σ^H (with 18 of these genes coregulated by σ^B, σ^H, and at least one additional regulator) and 31 genes regulated by both σ^B and σ^L (with 10 of these genes coregulated by σ^B, σ^L, and at least one additional regulator). Comparative phenotypic characterization measuring acid resistance, heat resistance, intracellular growth in J774 cells, invasion into Caco-2 epithelial cells, and virulence in the guinea pig model indicated contributions of (i) σ^B to acid resistance, (ii) CtsR to heat resistance, and (iii) PrfA, σ^B, and CtsR to virulence-associated characteristics. Loss of the remaining transcriptional regulators (i.e., *sigH*, *sigL*, or *sigC*) resulted in limited phenotypic consequences associated with stress survival and virulence. Identification of overlaps among the regulons provides strong evidence supporting the existence of complex regulatory networks that appear to provide the cell with regulatory redundancies, along with the ability to fine-tune gene expression in response to rapidly changing environmental conditions.

Pathogenic bacteria use a diverse set of strategies to survive conditions encountered in the environment and in the host, including a number of mechanisms that act at the level of transcriptional regulation. One important regulatory mechanism in bacteria that enables transcription of a targeted set of genes under appropriate environmental conditions is mediated through differential associations between various alternative σ factors and core RNA polymerase (45, 63). The food-borne bacterial pathogen *Listeria monocytogenes* has four alternative sigma factors (σ^B, σ^C, σ^H, and σ^L) (22). The general stress-responsive σ^B, encoded by *sigB*, is the most extensively characterized of the four alternative sigma factors (3, 8, 13–15, 70). To date, the σ^B regulon has been shown to include more than 150 genes in *L. monocytogenes* 10403S (58) and more than 100 genes in *L. monocytogenes* EGD-e (26). Global analysis of a *sigL* (*rpoN*) mutant (*sigL* encodes σ^L or σ^{54}) in *L. monocytogenes* EGD-e identified 20 genes as positively regulated by σ^L (1), and a *sigL* mutant previously showed impaired growth relative to the wild-type strain at low temperature, in the presence of salt, and under lactic acid stress (59). An *L. monocytogenes sigH* mutant has shown reduced growth in minimal medium and under alkaline conditions compared to the wild-type strain (61). σ^C, an extracytoplasmic function (ECF) sigma factor present only in *L. monocytogenes* strains classified in lineage II, is activated upon heat stress (72). The transcriptional repressors CtsR and HrcA negatively regulate heat shock genes (e.g., *clpP*, *clpC*, *dnaK*, and *groES*) and thus contribute to heat stress resistance with a *ctsR* null mutant showing increased heat resistance (27, 33, 53). Emerging evidence of transcriptional networks among σ^B and the negative regulators CtsR and HrcA (29, 30) suggests that *L. monocytogenes* mounts a coordinated response to various environmental stresses.

Perhaps the most rigorously supported example of overlapping and complementary interactions between *L. monocytogenes* transcriptional regulators exists between σ^B and the master virulence gene regulator, PrfA (5, 20, 36, 49, 56, 69). PrfA regulates the genes comprising the 10-kb *L. monocytogenes* virulence gene locus (*prfA-plcA-hly-mpl-actA-plcB*) in addition to other virulence genes located elsewhere on the chromosome (e.g., *inlA*, *inlB*, and *inlC*, which all encode internalins). One of the 3 promoters upstream of *prfA* ($P2_{prfA}$) is σ^B dependent, indicating a direct regulatory link between σ^B and PrfA (52, 60, 64). Some virulence genes (e.g., *inlA*, *inlB*, and *bsh*) are preceded by both PrfA boxes and σ^B promoters and appear to be coregulated by PrfA and σ^B (34, 50, 69).

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TABLE 1. *L. monocytogenes* strains used in this study

Strain or plasmid	Genotype	Details of construction[a] and/or reference
pSC9	pLIV2-*sigC* (IPTG-inducible P_{spac} promoter)	This study
pBMB47	pBMB6 P_{mcsA}-*gus*	This study
pBMB49	pBMB6 P_{hrcA}-*gus*	This study
10403S	Parent strain (serotype 1/2a)	4
FSL C3-126	10403S Δ*sigH*	Deleted 591 of 606 nt in *sigH* ORF (6)
FSL B2-124	10403S Δ*sigL*	Deleted 1,236 of 1,344 nt in *sigL* ORF (6)
FSL C3-113	10403S Δ*sigC*	Deleted 507 of 549 nt in *sigC* ORF (6)
FSL C3-143	10403S $tRNA^{Arg}$::pLIV2-*sigC*	pSC9 → 10403S
FSL A1-254	10403S Δ*sigB*	Deleted 297 of 780 nt in *sigB* ORF (70)
FSL B2-046	10403S Δ*prfA*	Deleted 339 of 714 nt in *prfA* ORF (71)
FSL H6-190	10403S Δ*ctsR*	Deleted 447 of 459 nt in *ctsR* ORF (30)
FSL H6-195	H6-190 $tRNA^{Arg}$::pLIV2-*ctsR-mcsA*	30
FSL B2-101	10403S Δ*hrcA*	Deleted 744 of 1,038 nt in *hrcA* ORF (29)
FSL B2-142	10403S P_{mcsA}-*gus*	pBMB47 → 10403S
FSL B2-143	10403S Δ*sigB* P_{mcsA}-*gus*	pBMB47 → A1-254
FSL B2-144	10403S P_{hrcA}-*gus*	pBMB49 → 10403S
FSL B2-145	10403S Δ*sigB* P_{hrcA}-*gus*	pBMB49 → A1-254

[a] The arrow denotes transformation of a plasmid into the recipient strain.

The primary goal of this work was to characterize transcriptional networks in *L. monocytogenes* as they relate to the four alternative sigma factors (σ^B, σ^C, σ^H, and σ^L), the virulence gene regulator PrfA, and the heat shock-related negative regulators CtsR and HrcA. Multiple transcriptional approaches were applied (i.e., whole-genome microarrays, quantitative reverse transcriptase [RT] PCR, 5′-modified rapid amplification of cDNA ends [RACE]-PCR) using a set of 8 strains, including the 10403S parent strain and 7 mutant strains, each bearing a null mutation in a gene encoding a regulatory protein. These strategies provided novel insight into the σ^C, σ^H, and σ^L regulons, which, in combination with data describing the σ^B, CtsR, and HrcA regulons (29, 30, 58) and the PrfA regulon (50, 56; this study), was used to identify putative coregulated genes in *L. monocytogenes* 10403S. In addition, the null mutant strains were phenotypically characterized in a series of assays to determine the relative contributions of these transcriptional regulators to acid resistance, heat resistance, intracellular growth in J774 cells, invasion into Caco-2 epithelial cells, and virulence in the guinea pig model. Cumulative data from transcriptomic analyses and phenotypic characterization strongly suggest overlapping regulons that create regulatory networks among these transcriptional regulators.

MATERIALS AND METHODS

Bacterial strains and mutant construction. *L. monocytogenes* 10403S (4) and 7 isogenic mutant strains, namely, the Δ*sigB* (70), Δ*sigC*, Δ*sigH*, Δ*sigL*, Δ*ctsR* (30), Δ*hrcA* (29), and Δ*prfA* (71) strains, were used in this study (Table 1). Δ*sigC*, Δ*sigH*, and Δ*sigL* strains in an *L. monocytogenes* 10403S background were constructed using splicing by overlap extension (SOE) PCR and allelic exchange mutagenesis, as detailed previously (6); all mutations were confirmed by PCR and sequencing of the chromosomal copy of the deletion allele. In addition, a 10403S strain expressing *sigC* from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{spac} promoter was constructed, as described by Hu et al. (30), by inserting pLIV2 with a *sigC* open reading frame (ORF) (see Table S1 in the supplemental material for primers used in cloning) into the 10403S $tRNA^{Arg}$ gene, yielding 10403S $tRNA^{Arg}$::pLIV2-*sigC* (Table 1).

Chromosomal transcriptional fusion strains were created to confirm σ^B-dependent expression of *hrcA* and *mcsA* (Table 1; see also Table S1 in the supplemental material), as described by Ferreira et al. (15). Specifically, hidden Markov model (HMM)-identified σ^B-dependent promoters for either *mcsA* or *hrcA* were cloned upstream of the *gus* gene in pBMB6 (64) to create pBMB47 and pBMB49, which contain P_{mcsA}-*gus* and P_{hrcA}-*gus*, respectively. The resulting plasmids were electroporated into both the *L. monocytogenes* 10403S and 10403S Δ*sigB* strains, followed by selection for strains with chromosomally integrated plasmids. Plasmid integrations and *gus* fusions were confirmed by PCR.

Microarray construction and design. Microarray experiments were performed as described in reference 7 using the Cornell University Food Safety Laboratory (CUFSL) *Listeria* 6K oligonucleotide array (NCBI GEO GPL5029). DNA microarrays were constructed to include 70-mer oligonucleotides representing the following: (i) 2,857 ORFs from *L. monocytogenes* EGD-e (Operon Array-Ready Oligo Sets) and (ii) five *Saccharomyces cerevisiae* ORFS (*act1*, *mfa1*, *mfa2*, *ras1*, and *ste3*) as negative controls. Serial (1:2) dilutions of *L. monocytogenes* chromosomal DNA (isolated according to the method of Flamm et al. [16]) and salmon sperm DNA were included on the array as positive and printing quality controls, respectively.

Cell growth conditions for RNA isolation, cDNA labeling, and microarrays. The *L. monocytogenes* strain 10403S and Δ*sigC*, Δ*sigH*, Δ*sigL*, and Δ*prfA* strains were grown at 37°C with shaking (230 rpm) to stationary phase (defined in this study as growth to an optical density at 600 nm [OD_{600}] of 1.0, followed by incubation at 37°C for an additional 3 h) prior to RNA isolation; these growth conditions were used to ensure stationary-phase *L. monocytogenes* cultures, as previously described (19), and to be consistent with previous microarray studies (7, 29, 30, 56, 58). RNA isolation and subsequent microarray experiments were performed as described previously (29, 30, 58). For determination of the σ^C regulon, we also compared transcript levels of (i) 10403S and the Δ*sigC* strain, both grown to log phase (OD_{600} = 0.4) and then exposed to 50°C for 10 min, and (ii) 10403S $tRNA^{Arg}$::pLIV2-*sigC* and the Δ*sigC* strain, using bacteria grown to log phase (OD_{600} = 0.4) in brain heart infusion (BHI) with 0.5 mM IPTG.

For each microarray comparison, at least three independent cell collections and RNA isolations were performed. RNA isolation (using the RNAprotect bacterial reagent and the RNeasy Midi kit [Qiagen, Valencia, CA]) and DNase treatment were performed as described in Raengpradub et al. (58). The SuperScript Plus Indirect cDNA labeling system for DNA microarrays (Invitrogen, Carlsbad, CA) was used according to the manufacturer's protocol to synthesize cDNA from total RNA and differentially label the cDNA. For each parent-null mutant comparison, purified, labeled cDNA for the parent strain and the corresponding null mutant strain (differentially labeled with fluorescent dyes) were combined, dried, and resuspended in 1× hybridization buffer prior to overnight hybridization at 42°C. Slide blocking, hybridization, posthybridization washing, and scanning were performed as previously described (47).

Statistical analysis of microarray data. Raw TIFF images of the microarrays were automatically gridded and analyzed using the GenePix Pro 6.0 software program (Molecular Devices, Sunnyvale, CA). Empty, saturated, and irregular spots were flagged and removed from subsequent analysis. The LIMMA software package (65), available from the R/BioConductor software project (21), was used for data preprocessing (background correction, as well as within-array and between-array normalization) and differential expression analysis (66). To account for duplicate spots, the "duplicateCorrelation" function was used (65). *P* values were adjusted for multiple comparisons by controlling for the false discovery

rate. Microarray data specifically generated for this article were submitted to the NCBI Gene Expression Omnibus (GEO) data repository under the accession numbers listed below. Microarray data for other mutant strains used here had previously been submitted under accession numbers GSE7492 (wild-type strain versus Δ*sigB* strain), GSE7514 (wild type versus the Δ*ctsR* strain and IPTG-inducible *ctsR* strain versus the Δ*ctsR* strain), GSE7517 (wild type versus the Δ*hrcA* strain), and GSE11347 (PrfA* strain versus Δ*prfA* strain).

Hidden Markov model (HMM)-based identification of putative σ^C-, σ^H-, and σ^L-dependent promoters. To identify putative σ^C-, σ^H-, σ^L-, and PrfA-dependent promoters, HMMs were built using the HMMER software program, version 2.3.2 (http://hmmer.janelia.org), with the HMMER null model settings modified to reflect the GC content of the *L. monocytogenes* genome (A/T = 0.31; G/C = 0.19; and p1 parameter = 0.966). Training sequences from known and putative *L. monocytogenes* and *Bacillus subtilis* promoters were used (as listed in Table S2 in the supplemental material). Separate models were built for the forward and reverse orientations. Outputs were filtered, and only hits within 750 bp upstream of a start codon for an ORF, as annotated by Listilist (http://genolist.pasteur.fr/ListiList), and with an E value of ≤0.1 were considered meaningful.

Analyses of σ factor, PrfA, HrcA, and CtsR regulons and regulon overlaps. To identify interactions and overlaps between different transcriptional regulators and their regulons, we performed comprehensive data analyses using the microarray-based data for the σ^C, σ^H, and σ^L regulons reported here together with previously reported microarray-based data for the σ^B (58), PrfA (56), HrcA (29), and CtsR (30) regulons. Putative σ^B-dependent promoters, CtsR-binding sites, and HrcA-binding sites were determined previously using HMM (29, 30, 58).

Gene set enrichment analysis (GSEA) (67) was used to identify gene sets that were significantly overrepresented among genes up- or downregulated in a given mutant strain; for some null mutant strains (e.g., the Δ*sigB* strain), GSEA analyses were performed separately for data generated using strains harvested under different conditions (e.g., salt stress or stationary phase). GSEA was run on the ranked list of $\log_2$ expression ratios obtained from the fitted normalized data in LIMMA with 1,000 permutations and exclusion of gene sets with fewer than 5 or greater than 500 members. Genes were classified into sets based on the TIGR Comprehensive Microbial Resource (http://cmr.tigr.org) subrole categories for *L. monocytogenes* EGD-e. False discovery rate *q* values less than 0.25 were considered significant.

Self-organizing tree algorithm (SOTA) clustering (28) was utilized to identify groups of genes that showed similar trends in gene expression across the data sets for different regulators. Log_2 expression ratios, obtained from the fitted normalized data in LIMMA for all of the comparisons (i.e., wild-type strain versus Δ*sigB* Δ*sigH*, Δ*sigL*, Δ*ctsR*, Δ*hrcA*, or Δ*prfA* strain and strain with IPTG-inducible *sigC* or *ctsR* versus Δ*sigC* or Δ*ctsR* strain, respectively), were assembled and filtered to identify all genes with a ≥1.5-fold expression difference between the wild-type and mutant strains for at least two of the comparisons. This approach identified 353 genes that were clustered using SOTA, implemented in the MeV 4.0 software program (www.tigr.org), with Pearson's correlation, 12 cycles, and a maximum cell diversity of 0.9.

Transcriptional fusion reporter assays. Transcriptional fusion strains were grown under two conditions that induce σ^B activity, including (i) late stationary phase (18 h of growth) or (ii) log phase (i.e., OD_{600} of approximately 0.4) followed by exposure to 0.3 M NaCl in BHI for 2 h. For the β-glucuronidase (GUS) assays, 1 ml of culture was harvested, washed twice with AB light buffer, and frozen in liquid nitrogen. Cells were resuspended in AB light and lysed using CelLytic B 2X reagent (Sigma-Aldrich, St. Louis, MO), and after centrifugation, the resulting supernatant was used in GUS assays as described previously (15, 64), with minor modifications. Briefly, 20 μl of supernatant and 60 μl of AB light-dimethyl sulfoxide (DMSO) were transferred into a black flat-bottom 96-well plate (Corning Incorporated, Corning, NY). A 20-μl aliquot of 0.4-mg/ml 4-methylumbelliferyl β-D-glucuronide (4-MUG) was added to each well. After a 60-min incubation in the dark, fluorescence was read using a Fusion plate reader (Perkin Elmer) with an excitation wavelength of 365 nm and emission wavelength of 460 nm. A standard curve was created for the fluorescent product, 4-methylumbelliferone (MU). GUS activities were determined and reported as pmol of MU generated per min per OD_{600} of the bacterial culture.

RACE-PCR and TaqMan quantitative RT-PCR. RNA for rapid amplification of cDNA ends (RACE) and quantitative RT-PCR (qRT-PCR) was isolated and DNase treated using the same protocol as for the microarray experiments. Primers and probes used in these experiments are listed in Table S1 in the supplemental material. For RACE-PCR, the 5′ RACE system from Invitrogen (Carlsbad, CA) was used to map the transcriptional start sites for *mcsA* and *hrcA* (34). Briefly, cDNA was synthesized using a gene-specific primer (GSP1) and tailed with dCTP using terminal deoxynucleotidyl transferase (TdT). Poly(dC)-tailed cDNA was amplified using a nested gene-specific primer (GSP2; located 5′ to GSP1) and a poly(G/I) primer using AmpliTaq Gold polymerase (Applied Biosystems) and a touchdown PCR protocol, which incorporates a 0.5°C decrease in annealing temperature in each of the first 20 cycles. The specific PCR condition included the following: (i) one cycle at 95°C for 4 min, 30 s; (ii) 20 cycles of 95°C for 30 s, annealing (for 30 s) at 58°C, decreasing to 48°C (at 0.5°C decrease per cycle), and 72°C for 1 min; (iii) another 20 cycles of 95°C for 30 s, 48°C for 30 s, and 72°C for 1 min; and (iv) a final cycle of 72°C for 7 min. PCR products were separated on 3% high-definition agarose gels, and bands of interest were gel extracted and purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA). RACE-PCR products were cloned into pCR2.1 using the Topo TA cloning kit (Invitrogen), and plasmid inserts were sequenced.

To confirm the σ^C regulon, TaqMan probe and primer sets were designed, using the Primer Express 1.0 software program (Applied Biosystems, Foster City, CA), for the genes lmo0402, lmo0422, lmo0426, lmo1849, and lmo2648. Further qRT-PCR assays were performed to confirm key cross-regulation between different regulators identified in the initial microarray experiments (i.e., instances where one transcriptional regulator regulates another gene that encodes a regulator); in addition to previously reported (29, 30, 34) TaqMan primers and probes for *clpC* (lmo0232) and *groES* (lmo2069), we also designed new primers and probes for *sigH*, *sigC*, *hfq* (lmo1295), *hrcA*, and *cggR* (lmo2460) for these experiments. qRT-PCR was performed as described in reference 36 using TaqMan One-Step RT-PCR master mix reagent, Multiscribe reverse transcriptase (RT), and the ABI Prism 7000 sequence detection system (Applied Biosystems). Genomic DNA standard curves were generated for each gene. All statistical analyses were performed on log-transformed ($\log_{10}$) numbers of mRNA transcript levels normalized to the geometric mean for *rpoB* and *gap* mRNA transcript levels obtained under the same experimental conditions. The general linear model (GLM) procedure (F test) was used to compare log-transformed values with strain and replicate as factors. For multiple comparisons, a Tukey-Kramer adjustment was applied.

Acid and heat stress survival assays. *L. monocytogenes* 10403S and the 7 mutant strains were assessed for their abilities to survive heat stress (55°C for 1 h) or acid stress (pH 2.5 for 1 h) as described in Hu et al. (29). For both assays, strains were grown to early stationary phase (defined as growth to an OD_{600} of 0.8, followed by an additional 1 h of incubation) in BHI at 37°C; this growth condition was selected to allow comparison with previous acid and heat stress survival assays that were performed using the same conditions (29). Briefly, for heat stress, early-stationary-phase cells were exposed to 55°C in a water bath for 1 h. For acid stress, the culture pH was adjusted to 2.5 using 12 N HCl. Samples were harvested after 1 h of exposure. Bacterial numbers prior to and after stress treatments were enumerated on BHI agar plates using an Autoplate 4000 spiral plater (Spiral Biotech Inc., Norwood, MA). Changes in bacterial numbers were reported as log CFU/ml reduction, which was calculated as (log CFU/ml prestress) − (log CFU/ml poststress).

Invasion assay. The human colorectal adenocarcinoma epithelial cell line Caco-2 (ATCC HTB-37) was cultured, and invasion assays were performed as described by Garner et al. (20) with minor modifications. Briefly, 5.0×10^4 Caco-2 cells were seeded into 24-well plates (Corning Incorporated, Corning, NY) 48 h prior to infection. For infection, the Caco-2 cells were inoculated with approximately 2×10^7 *L. monocytogenes* cells grown to early stationary phase. *L. monocytogenes* numbers used for infection were verified by plating on BHI agar. Intracellular *L. monocytogenes* numbers were determined 90 min postinfection. Invasion efficiency was calculated as the number of bacteria recovered relative to the number of bacteria used for inoculation (i.e., [log CFU/ml recovered] − [log CFU/ml inoculated]).

Intracellular growth assay. The mouse macrophage-like cell line J774A.1 (ATCC TIB-67) was cultivated at 37°C with 5% CO_2 in J774 medium (Dulbecco's modified Eagle medium with Earle's salts and 1% sodium pyruvate [Gibco, Gaithersburg, MD] containing 10% fetal bovine serum [Gibco], 1.5 g/liter sodium bicarbonate [Gibco], and 100 μg/ml [each] penicillin G and streptomycin). At approximately 48 h before intracellular growth assays, J774 cells were seeded at a density of 2×10^5 cells/ml in each well of a 24-well plate using J774 medium without antibiotics. To activate macrophages, at approximately 24 h before infection, J774 cells were shifted to medium (without antibiotics) that contained lipopolysaccharide (LPS) (Sigma, St. Louis, MO) at a final concentration of 100 ng/ml. At 30 min prior to infection, fresh medium (without antibiotics) was added. J774 cells were then inoculated with *L. monocytogenes* at a multiplicity of infection (MOI) of 1. *L. monocytogenes* used for inoculation had been grown to early stationary phase in BHI, flash frozen, and stored in liquid nitrogen before the assays. Bacterial numbers were verified by plating on BHI agar immediately prior to the assays. At 30 min postinoculation (p.i.), J774 cells were washed with 1 ml sterile phosphate-buffered saline (PBS), followed by addition of 1 ml of fresh medium with 50 μg/ml gentamicin. At 1.5, 3.5, 5.5, and 7.5 h p.i., wells were

washed three times with 1 ml of sterile PBS and lysed with 500 μl of ice-cold sterile distilled water, followed by plating of the cell suspension on BHI agar to determine intracellular bacterial numbers at each time point. Intracellular growth was calculated as the number of bacteria recovered at each time point x (x = 3.5, 5.5, and 7.5 h) relative to the number of bacteria recovered at T = 1.5 h (i.e., [log CFU/ml $T = x$] − [log CFU/ml T = 1.5]); bacterial numbers at T = 1.5 h represent an estimate of the initial intracellular bacterial numbers and were thus used as a baseline value for starting bacterial numbers. With this approach, positive log CFU/ml values at a given time point signify intracellular growth.

Animal care and housing. Animal protocols (2002-0060) were approved by the Cornell University Institutional Animal Care and Use Committee prior to initiation of the experiments. Male Hartley guinea pigs (Elm Hill, Chelmsford, MA) weighing approximately 300 g at about 3 weeks of age were housed individually. Animals were provided with feed and water *ad libitum*. Cages were changed daily, and animal health and weight were monitored and recorded daily.

Intragastric infection of guinea pigs. Intragastric infections of guinea pigs were performed as described previously (20, 42) with some modifications. Briefly, food was removed from cages 12 h prior to infection. Animals were weighed and then anesthetized with isoflurane administered via inhalation. Each *L. monocytogenes* strain (wild type or the Δ*sigH*, Δ*sigL*, Δ*sigC*, Δ*ctsR*, or Δ*hrcA* strain) was inoculated intragastrically (at a dose of 1×10^{10} CFU) after stomach pH was buffered by administering 1.5 ml of PBS containing 125 mg calcium carbonate (pH 7.4); negative-control guinea pigs were administered PBS after buffering the stomach pH. Animal weights were measured daily. At 72 h p.i., animals were euthanized via carbon dioxide inhalation. For each strain, 4 guinea pigs were inoculated; a total of 4 uninoculated guinea pigs were included as controls.

Enumeration of *L. monocytogenes* from organs. Organs were harvested at 72 h p.i. as previously described by Lecuit et al. (42). The liver, mesenteric lymph nodes, and small intestine (a 20-cm portion, immediately proximal to the cecum) were aseptically removed and processed for *L. monocytogenes* enumeration essentially as described by Garner et al. (20); confirmation of *L. monocytogenes* on *L. monocytogenes* plating medium (LMPM) was performed only if negative controls showed *Listeria*-like colonies on Oxford agar. To detect *L. monocytogenes* present in a given organ at below the detection limit of direct plating, 10 ml of each organ homogenate was added to 90 ml of *Listeria* enrichment broth (Becton Dickinson, Sparks, MD), followed by incubation at 30°C for 48 h. Samples exhibiting colonies with *Listeria*-like morphology after plating on Oxford agar (Oxoid, Ogdensburg, NY) and subsequent incubation at 30°C for 48 h were recorded as *L. monocytogenes* "enrichment positive."

Enumeration of *L. monocytogenes* from feces. Fecal pellets from each animal were collected daily, and *L. monocytogenes* was enumerated according to the method of Garner et al. (20). After incubation at 30°C for 48 h, colonies exhibiting *Listeria*-like morphology on Oxford agar were counted and recorded as *L. monocytogenes*; confirmation of *L. monocytogenes* on LMPM was performed only if negative controls showed *Listeria*-like colonies on Oxford agar.

Statistical analyses of phenotypic characterizations. Statistical analyses for differences among strains with regard to stress survival (heat and acid), invasion efficiency, intracellular growth, and infection in the guinea pig model (i.e., log CFU/g in liver, spleen, small intestine, and mesenteric lymph nodes, change in guinea pig weight after inoculation, and fecal *L. monocytogenes* counts) were performed using GLM with Dunnett's t test.

Microarray data accession number. Microarray data specifically generated for the manuscript and not previously reported were submitted to the GEO data repository under accession no. GSE24339; this submission includes microarray data for the wild type versus the Δ*sigL* strain, the wild type versus the Δ*sigH* strain, the wild type versus the Δ*sigC* strain (all generated using stationary-phase bacteria), a strain with an IPTG-inducible *sigC* gene versus the Δ*sigC* strain, and the wild type versus the Δ*sigC* strain (using cells grown to log phase and exposed to heat stress).

RESULTS

σ^H regulon. Whole-genome microarray analyses using a Δ*sigH* strain and an otherwise isogenic 10403S parent strain grown to stationary phase were used to identify members of the *L. monocytogenes* σ^H regulon. Using an adjusted P value of <0.05 and a 1.5-fold change as cutoff values, we identified 97 genes that were positively regulated by σ^H (i.e., with higher transcript levels in the parent than in the mutant strain), including 56 genes likely to be directly regulated by σ^H, as supported by HMM identification of a putative σ^H-dependent promoter upstream of a given gene or the first gene in a predicted operon (Table 2; see also Table S3 in the supplemental material). A position-weight matrix analysis of the σ^H consensus promoter showed the conserved sequences AGG and GAA at the −35 and −10 regions, respectively (see Fig. S2A in the supplemental material). We also identified 72 genes that showed significantly higher transcript levels in the Δ*sigH* strain than in the parent, suggesting either upregulation to compensate for genes that are positively regulated by σ^H (and thus downregulated in the Δ*sigH* strain) or indirect negative regulation of these genes by σ^H (e.g., through σ^H-dependent transcription of other regulators, such as lmo2190, which encodes the negative regulator MecA, or lmo2690, which encodes a protein similar to TetR [both of these genes were found to be σ^H dependent in our microarray data]). Gene set enrichment analyses (GSEA) of the σ^H regulon did not identify any specific gene sets that were enriched among positively regulated genes but identified a number of subrole categories enriched among the negatively regulated genes (Table 3).

σ^L regulon. To characterize the σ^L regulon, whole-genome microarray experiments were conducted using the Δ*sigL* strain and the otherwise isogenic parent strain grown to stationary phase. Using the same statistical criteria as for the σ^H regulon characterization above, we identified 31 genes that were positively regulated by σ^L, including 16 genes likely to be directly regulated by σ^L, as supported by HMM identification of a putative σ^L-dependent promoter upstream of a given gene or the first gene in a predicted operon (Table 2; see also Table S4 in the supplemental material). A position-weight matrix of the σ^L consensus promoter identified conserved GG and GC sequences at the predicted −24 and −12 regions (see Fig. S2B). A specific example of genes positively regulated by σ^L includes the lmo0398-lmo0402 operon (with transcript level fold changes ranging from 3.4 to 19.2) (see Table S4). Genes in this operon are involved in carbohydrate utilization and encode components of a fructose-specific phosphotransferase system (PTS), a glycosyl hydrolase, and a BglG family transcriptional terminator. Twenty genes showed significantly higher transcript levels in the Δ*sigL* strain than in the parent strain, suggesting negative regulation of these genes by σ^L. GSEA of the σ^L regulon identified in the present study established a number of subrole categories enriched among genes positively regulated by σ^L (with most classified into the "energy metabolism" role category; Table 3) as well as a number of subrole categories enriched among genes negatively regulated by σ^L (Table 3).

Since a previous report identified 20 and 57 genes, respectively, as positively and negatively regulated by σ^L in another *L. monocytogenes* strain (i.e., strain EGD-e) (1), we also compared the σ^L regulon identified here to this previously reported regulon. The only overlap among the 51 10403S genes identified as σ^L regulated in the present study and the 77 EGD-e genes reported by Arous et al. (1) is a set of 4 genes determined in both studies to be negatively regulated by σ^L (see Table S4 in the supplemental material). Possible explanations for the lack of overlap include evaluation of different *L. monocytogenes* strains and use of different growth conditions between the two studies. Specifically, Arous et al. (1) created an insertional mutation in the EGD-e *sigL* gene and used bacte-

TABLE 2. Genes directly or indirectly regulated by PrfA, CtsR, HrcA, and the four alternative sigma factors[a]

Regulator (gene)	No. of genes showing[b]:				Reference
	Positive regulation		Negative regulation		
	With TFBS (indicating direct regulation)	Without TFBS (indicating indirect regulation)	With TFBS (indicating direct regulation)	Without TFBS (indicating indirect regulation)	
σ^H (lmo0243)[c]	56	41	NA[d]	72	This study
σ^L (lmo2461)[e]	16	15	NA	20	This study
σ^C (lmo0423)	0[f]	0	NA	3[g]	This study
σ^B (lmo0895)[h]	288	59	NA	420	58
PrfA (lmo0200)[i]	13	3	NA	5	56
CtsR (lmo0229)[j]	NA	21	1	24	30
HrcA (lmo1475)[k]	NA	34	8	17	29

[a] Genes were considered differentially regulated when the microarray comparison between the parent strain and the mutant yielded a fold change (rounded to one significant digit) ≥ 1.5 and an adjusted *P* value (rounded to 3 significant digits) < 0.05. To ensure that all comparisons were conducted on data sets that had been analyzed in a consistent manner, previously published data for the sigma B (58), HrcA (29), and CtsR (30) regulons were reanalyzed using the rounding strategies and cutoff values established for the present study. As a consequence, the numbers of genes reported here for the sigma B, HrcA, and CtsR regulons differ slightly from those previously published.

[b] The presence or absence of a relevant upstream HMM-predicted sequence or transcription factor binding site (TFBS) was used to classify each gene as "directly" or "indirectly" regulated, respectively.

[c] All σ^H-dependent genes are detailed in Table S3 in the supplemental material.

[d] NA, not applicable; for genes that show evidence (i) for negative regulation in a null mutant where a positive regulator (i.e., PrfA or an alternative sigma factor) was deleted or (ii) for positive regulation in a null mutant where a negative regulator (i.e., CtsR and HrcA) was deleted, the presence of a putative transcription factor binding site is not relevant and hence is not reported.

[e] All σ^L-dependent genes are detailed in Table S4 in the supplemental material.

[f] No genes showed evidence for positive regulation by σ^C in the microarray data (using a fold change cutoff of 1.5 and an adjusted *P* value cutoff < 0.05); genes that were at least 1.5-fold differentially expressed in the parent strain relative to the mutant strain, but with an adjusted *P* value > 0.05, are detailed in Table S5 in the supplemental material.

[g] The 3 genes that are negatively regulated by σ^C include lmo1854 and the lmo2185-lmo2186 operon (see Table S5 in the supplemental material).

[h] Genes recognized as σ^B dependent either under salt stress or in stationary-phase cells. Genes that differed in the direction of σ^B-dependent expression (e.g., upregulated in salt stress and downregulated in stationary phase) under these conditions (i.e., lmo0850, lmo1293, lmo1335, lmo1473, lmo1539, lmo1678, lmo1849, lmo1993, lmo2040, lmo2159, lmo2161, lmo2163, lmo2193, lmo2335, lmo2557, lmo2597, and lmo2785) were not included. The number of σ^B-dependent genes differs from the previous report (58) due to different statistical cutoff criteria used in the study reported here (adjusted $P < 0.05$ and a fold change ≥ 1.5 were used here to be consistent for all regulators). σ^B itself is autoregulated but was not included in the 288 genes that are listed as directly positively regulated. A complete list of σ^B-dependent genes as identified by microarray with the cutoff being an adjusted *P* value < 0.05 and a fold change ≥ 1.5 is shown in Table S6 in the supplemental material.

[i] Since no genes were found to be significantly differentially regulated in the comparison between 10403S and its isogenic Δ*prfA* strain (with the cutoff being an adjusted *P* value < 0.05 and a fold change ≥ 1.5), genes found to be positively and negatively regulated by PrfA* (as reported by Ollinger et al. [56]) are listed here. However, to provide analyses with the same parent strain, microarray data for the comparison between 10403S and its isogenic Δ*prfA* mutant were used for GSEA and SOTA analyses (see Table 3 and Fig. 1; see also Table S9 in the supplemental material). A total of 13 genes showed significantly higher transcript levels in the presence of PrfA* than in the absence of PrfA* (as determined by two-way analysis of variance [ANOVA]), while 4 genes showed significant interaction effects between σ^B and PrfA*, with higher transcript levels in the presence of PrfA*. lmo0090 was reported to be positively regulated by PrfA* in an *L. monocytogenes* 10403S background; however, it was confirmed by TaqMan RT-PCR to be PrfA* independent. Four genes (i.e., *ftsH*, *gltX*, *cysS*, and lmo0208) were reported to be negatively regulated by PrfA* (56); however, *ftsH* and *gltX* were shown by TaqMan RT-PCR not to be negatively regulated by PrfA*. Therefore, only *cysS* and lmo0208 are considered to be PrfA* dependent.

[j] Genes recognized as CtsR dependent either in log-phase cells or in a strain with an IPTG-inducible *ctsR* gene (30). The number of CtsR-dependent genes reported differs from previous report (30) due to different statistical cutoff criteria used in the study reported here.

[k] The number of HrcA-dependent genes reported differs from a previous report (29) due to application of different statistical cutoff criteria in the study reported here. While autoregulated, *hrcA* is not included among the HrcA-dependent genes listed here, and thus, only 8 genes, rather than the 9 genes previously reported (29), are listed as directly and negatively regulated by HrcA.

rial cells grown at 42°C in the presence of erythromycin (to maintain the plasmid in the chromosome) to early stationary phase (OD_{600} = 0.8 to 1.0), while the present study uses an in-frame *sigL* deletion in strain 10403S with growth of cells at 37°C in BHI to stationary phase (OD_{600} = 1.0, followed by incubation for an additional 3 h).

σ^C regulon. Initial characterization of the σ^C regulon using stationary-phase bacteria failed to identify any genes as σ^C dependent (using an adjusted *P* value of <0.05 and a 1.5-fold change as cutoff values). Additional microarray experiments were thus performed comparing transcript levels for the following: (i) 10403S and the Δ*sigC* strain, both grown to log phase (OD_{600} = 0.4) and then exposed to 50°C for 10 min (since Zhang et al. [72] suggested a role for σ^C in heat stress), and (ii) 10403S $tRNA^{Arg}$::pLIV2-*sigC* and the Δ*sigC* strain, using bacteria grown to log phase (OD_{600} = 0.4) in BHI with 0.5 mM IPTG (to overexpress *sigC* in the 10403S $tRNA^{Arg}$::pLIV2-*sigC* strain). Analyses of these microarray data (using an adjusted *P* value of <0.05 and a 1.5-fold change as cutoff values) identified only 3 σ^C-dependent genes (i.e., lmo1854 [with a higher transcript level in the Δ*sigC* strain than in the wild type after heat exposure] and the lmo2186-lmo2185 operon [with a higher transcript level in the Δ*sigC* strain than in the 10403S $tRNA^{Arg}$::pLIV2-*sigC* strain]), suggesting negative regulation of these genes by σ^C. To more fully explore possible members of the σ^C regulon, qRT-PCR was used to examine a subset of genes that showed a ≥1.5-fold difference in transcript levels (regardless of *P* values), with lower relative transcript levels in the Δ*sigC* strain in at least one of the three microarray comparisons. A total of 21 genes met this criterion (see Table S5 in the supplemental material), including 11 genes with putative σ^C-dependent promoters upstream of a given gene or the first gene in a predicted operon that had been identified using HMM. The 11 selected genes repre-

TABLE 3. GSEA results by biological function

Biological function[a]	Regulation by[b]:								
	SigH	SigL	*i*-SigC[c]	SigB-Salt[c]	SigB Stat[c]	PrfA	CtsR	*i*-CtsR[c]	HrcA
Amino acid biosynthesis									
Aspartate family	▼					▲			
Glutamate family, pyruvate family					▼				
Biosynthesis of cofactors, prosthetic groups, and carriers									
Heme, porphyrin, and cobalamin	▼								
Menaquinone and ubiquinone, molybdopterin	▼						▲		
Cell envelope									
Degradation of surface polysaccharides and lipopolysaccharides	▼								
Murein sacculus and peptidoglycan	▼	▼		▼					
Other					▲				
Cellular processes									
Adaptation to atypical conditions		▼		▲	▲				
Cell division	▼	▼				▲			
Chemotaxis and motility	▼	▼	▼	▼	▼	▲	▲		▲
Detoxification				▲	▲	▼		▲	
Pathogenesis				▲					
DNA metabolism									
DNA replication, recombination, and repair					▼				
Energy metabolism									
ATP proton-motive force interconversion						▲			
Biosynthesis and degradation of polysaccharides		▲			▲	▼	▲		
Electron transport		▲							
Fermentation		▲					▲	▲	
Glycolysis/gluconeogenesis			▲		▲	▼			
Pentose phosphate		▲			▲	▼			
Sugars							▲	▲	
TCA cycle[d]		▲	▼			▼			
Other						▼			
Mobile and extrachromosomal element functions									
Prophage functions								▲	
Protein fate									
Degradation of proteins, peptides, and glycoproteins							▼		
Protein folding and stabilization					▼				▼
Protein modification and repair					▼				
Protein synthesis									
Ribosomal protein synthesis and modification	▼	▼		▼	▼	▲		▼	▲
Translation factors	▼								▲
tRNA aminoacylation	▼				▼	▲			
tRNA and rRNA base modification	▼								
Purines, pyrimidines, nucleosides, and nucleotides									
Purine ribonucleotide biosynthesis	▼			▼					
Pyrimidine ribonucleotide biosynthesis	▼								
Salvage of nucleosides and nucleotides	▼			▼					
Transport and binding proteins									
Amino acids, peptides, and amines				▲					
Carbohydrates, organic alcohols, and acids		▲					▲		
Cations, unknown substrate					▼				
Viral functions									
General	▼								

[a] JCVI "main role categories" (representing more general groups, e.g., amino acid biosynthesis) are shown in bold, while JCVI "subrole categories" are listed below the main role categories; statistical analyses were performed only for the subrole categories.

[b] "▼" and "▲" indicate that a subrole category is overrepresented among genes negatively or positively regulated, respectively, by a given regulator, using a false discovery rate (FDR) cutoff with a q value <0.25.

[c] Salt, salt stress condition (0.3 M NaCl); Stat, stationary phase; "*i*-" indicates that the gene encoding a regulator was overexpressed using an IPTG-inducible promoter in pLIV2, which was integrated at the $tRNA^{Arg}$ site.

[d] TCA, tricarboxylic acid.

TABLE 4. Genes identified by microarray as coregulated by two transcriptional regulators only[a]

Coregulators	No. of genes coregulated[b]	No. of genes with a coregulation pattern of[c]:			
		▲▲	▲▼	▼▲	▼▼
σ^B, σ^H	74	39	1	5	29
σ^B, CtsR	25	2	6	6	11
σ^B, HrcA	21	1	5	11	4
σ^B, σ^L	21	11	2	–	8
σ^B, PrfA	10	3	2	3	2
σ^H, σ^L	3	1	–	1	1
σ^H, HrcA	3	2	–	1	–
σ^B, σ^C	2	–	–	–	2
σ^H, CtsR	2	–	1	1	–
CtsR, HrcA	2	–	1	1	–

[a] A total of 188 genes showed transcript levels that were dependent on 2 or more regulators, including 163 genes that were identified as coregulated by only two regulators (listed here; gene names are provided in Tables S7 and S8 in the supplemental material) and 25 genes identified as coregulated by 3 or more regulators (see Tables S7 and S8 in the supplemental material). All included genes met the cutoff criteria of an adjusted *P* value <0.05 and a fold change ≥ 1.5 for a given regulator; using these cutoff criteria, 39, 13, 0, 264, and 9 genes, respectively, were found to be upregulated by only σ^H, σ^L, σ^C, σ^B, and PrfA, and 14 and 10 genes, respectively, were found to be downregulated by only CtsR and HrcA.

[b] For sigma B, genes were classified as upregulated or downregulated if they had higher or lower transcript levels, respectively, in the parent strain than in the Δ*sigB* strain either under salt stress, in stationary phase, or under both conditions. Seventeen genes that differed in the direction of σ^B-dependent expression depending on the condition (e.g., upregulated in salt stress and downregulated in stationary phase) are not included. For PrfA, since no genes were found to be significantly differentially regulated in the comparison between 10403S and its isogenic Δ*prfA* strain (with an adjusted *P* value <0.05 and a fold change ≥ 1.5), genes found to be positively and negatively regulated by PrfA* (as reported by Ollinger et al. [56]) are thus reported here.

[c] ▲, higher transcript levels in wild type; ▼, higher transcript levels in null mutant; the order of symbols represents the order of regulators given in the first column; for example, "▲▼" for the first row indicates genes that are upregulated by σ^B and downregulated by σ^H.

sented 4 putative operons (lmo0400-lmo0402, lmo0425-lmo0426, lmo1849-lmo1848, and lmo2648-lmo2645). One gene in each predicted σ^C-dependent operon (lmo0402, lmo0426, lmo1849, and lmo2648), as well as a single gene with an apparent upstream σ^C-dependent promoter (lmo1698) and lmo0422, which is in the same operon as the *sigC* gene (lmo0423) itself, were chosen for transcript level analyses using qRT-PCR. Among these selected genes, only lmo0422 was verified as σ^C dependent under the conditions used (see Table S10).

Regulon overlaps and regulatory networks among transcriptional regulators. Microarray data and HMM-based promoter search data reported here for σ^H, σ^L, and σ^C were analyzed together with previously reported microarray and HMM data for the σ^B (58), PrfA (56), HrcA (29), and CtsR (30) regulons to comprehensively identify overlaps among regulons for different *L. monocytogenes* transcriptional regulators. In addition to a number of genes that were identified as regulated by only one of the regulators studied here (e.g., 39 and 13 genes, respectively, that were positively regulated by only σ^H and σ^L), initial analyses identified 188 genes that were regulated by more than one regulator (Table 4). A total of 163 genes were identified as coregulated by only two regulators (Table 4), while the remaining 25 genes were identified as being coregulated by 3 or more regulators (see Tables S7 and S8 in the supplemental material). A large number of genes (92 genes in total) appear to be regulated by both σ^B and σ^H, including 74 genes coregulated by σ^B and σ^H only (Table 4; see also Tables S7 and S8) and 18 genes coregulated by σ^B and σ^H and at least one additional regulator. In addition, a considerable overlap exists between σ^B- and σ^L-dependent genes, with 21 genes coregulated by σ^B and σ^L and 10 genes coregulated by σ^B, σ^L, and one or more additional regulators (Table 4; see also Table S7). Considerable overlap also was identified between σ^B and CtsR, including 25 genes coregulated by σ^B and CtsR only (Table 4; see also Table S7) and 12 genes coregulated by σ^B and CtsR and at least one additional regulator. Multiple coregulation patterns were identified within each overlapping regulon (e.g., among the 74 genes coregulated by σ^B and σ^H only, 39 or 29 genes, respectively, were up- or downregulated by both regulators [Table 4]). Each overlapping regulon also included genes categorized into multiple biological function role categories.

Self-organizing tree algorithm (SOTA) clustering was used to further characterize gene coregulation patterns based on microarray-generated data. Overall, SOTA analyses grouped genes into 13 different clusters of coregulated genes, based on regulation pattern (Fig. 1; see also Table S9 in the supplemental material), with a number of SOTA clusters representing genes that are coregulated by σ^B and σ^H (e.g., SOTA clusters 9, 10, and 13). The largest SOTA cluster (cluster 13, with 129 genes) included genes that are upregulated by σ^B, with some upregulated by σ^H, σ^L, and CtsR (which implies that CtsR can have an indirect, or positive, role in gene regulation). Genes in this cluster represented 15 different role categories, including 22 genes in energy metabolism and 18 genes in transport and binding (e.g., the *opucABCD* operon; see Table S9). Clusters 10 and 12 contained genes that are positively regulated by σ^B, σ^H, and σ^L but negatively regulated by PrfA, suggesting regulatory interactions among the virulence regulator PrfA and these alternative sigma factors. The majority of genes in these groups (i.e., 8 and 13 genes, respectively) are involved in energy metabolism. However, genes in cluster 12 are differentially regulated by σ^B depending on the environmental conditions affecting the cells (e.g., σ^B inhibits expression of the fructose phosphotransferase system lmo0398 operon under salt stress but positively regulates transcription of these genes during stationary phase). σ^B and CtsR were previously reported to coregulate some stress response and virulence genes, e.g., *clpC* (30).

GSEA showed that a number of subrole categories were enriched within multiple regulons (Table 3), including the "chemotaxis and motility" subrole category, which was overrepresented among genes negatively regulated by σ^H, σ^L, and σ^B and among genes positively regulated by PrfA, CtsR, and HrcA (Table 3). In agreement with GSEA results, SOTA clusters 5 and 7 (Fig. 1; see also Table S9 in the supplemental material), which contain genes that are negatively regulated by σ^H, σ^L, and σ^B and positively regulated by CtsR, include eight (of 28 total) chemotaxis and motility genes. Seven of the eight genes are located in two adjacent operons, i.e., lmo0675-lmo0689 and lmo0691-lmo0718. These findings suggest the importance of coordinated and differential regulation of *L. monocytogenes* motility under different conditions and are consistent with previous data that showed downregulation of motility and motility-regulated genes by σ^B (58, 69).

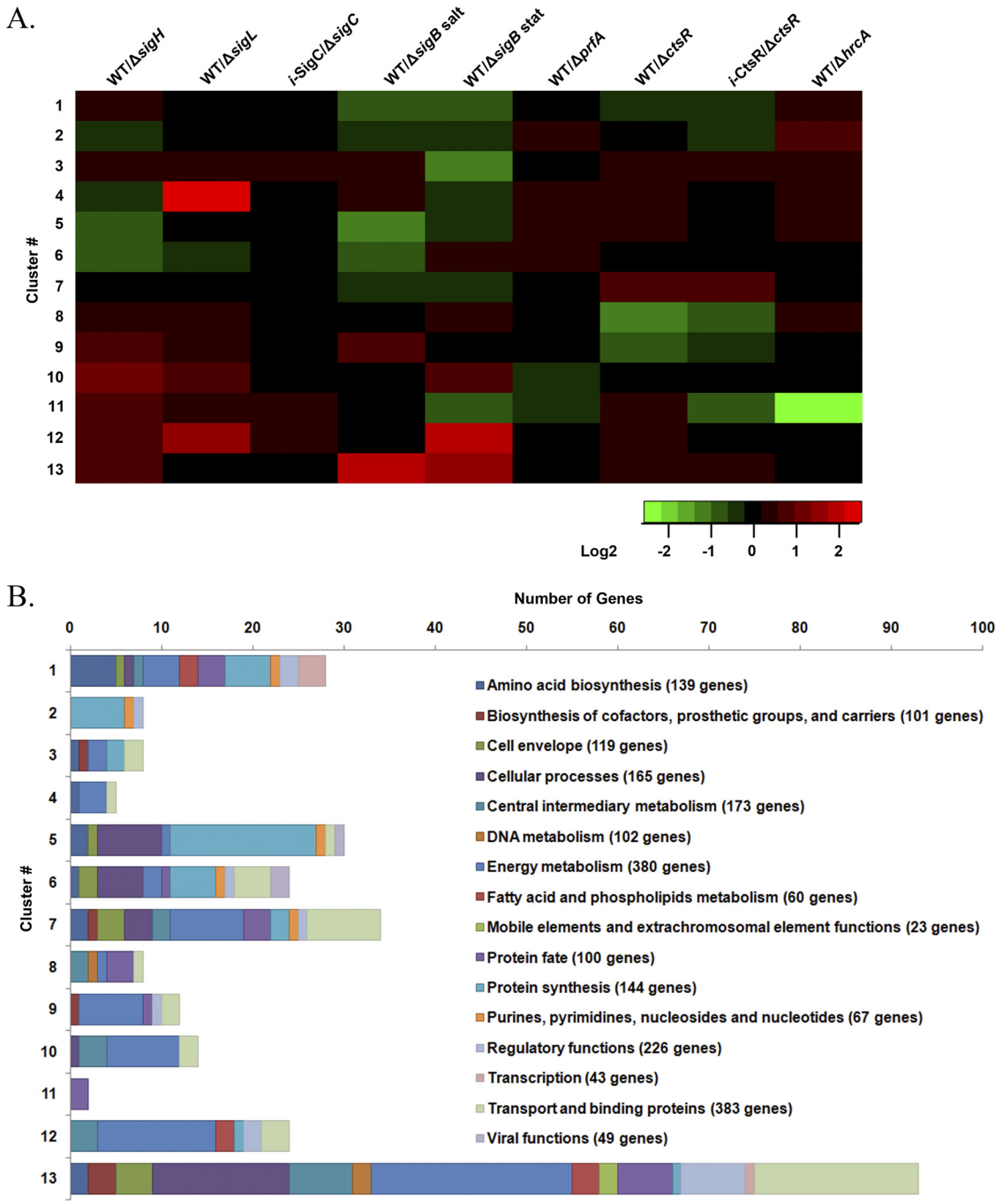

FIG. 1. Self-organizing tree algorithm (SOTA) clustering for genes with similar expression patterns. (A) Heat map of SOTA clusters. The average $\log_2$ expression ratio (parent/mutant) for genes in each SOTA cluster is represented as a heat map. Each row represents a SOTA cluster (labeled 1 to 13), and each column represents data from a parent-versus-mutant microarray comparison. Red clusters have a positive $\log_2$ expression ratio, which represents higher expression of genes in these clusters in the parent strain than in the mutant strain. Clusters that appear green have a negative $\log_2$ expression ratio, which represents higher expression of genes in these clusters in the mutant strain than in the parent strain, indicating that these genes are negatively regulated by that particular regulator. "*i*" indicates comparisons conducted with either *ctsR* or *sigC* expressed under the control of an IPTG-inducible promoter. (B) Number of genes in each SOTA cluster belonging to selected role category (total number of genes in a given role category is shown in parenthesis); genes classified in other role categories (e.g., hypothetical proteins, unknown function, unclassified, and unassigned) are not included here. Genes in each cluster are listed in Table S9 in the supplemental material.

Confirmation of transcriptional network connections and transcriptional patterns for selected coregulated genes. Analyses of microarray data identified a number of transcriptional patterns that suggested regulatory interdependencies among different transcriptional regulators and their regulons, including the following: (i) σ^B-dependent transcription of the *mcsA-mcsB-clpC* operon (which encodes regulators of CtsR activity), (ii) σ^B-dependent transcription of *hrcA*, and (iii) σ^L-dependent

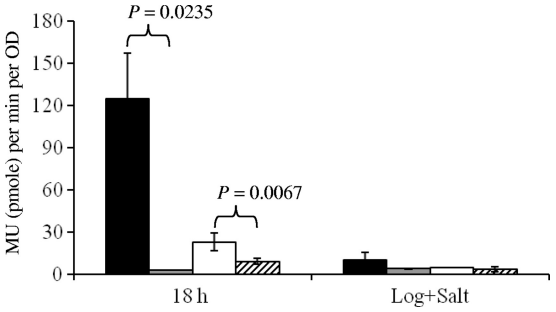

FIG. 2. σ^B-dependent GUS activities in 10403S P_{mcsA}-*gus* (black), the Δ*sigB* P_{mcsA}-*gus* strain (gray), 10403S P_{hrcA}-*gus* (white), and the Δ*sigB* P_{hrcA}-*gus* strain (hatched), grown either to late stationary phase (18 h) in BHI or to log phase (OD_{600} = 0.4) and exposed to 0.3 M NaCl for 2 h. Data shown represent the averages for three independent experiments; error bars indicate 1 standard deviation from mean values. *P* values are indicated where the differences are statistically significant.

transcription of the *sigC* operon. To confirm the regulatory interdependencies among different transcriptional regulators and the microarray-based evidence for transcriptional regulation of other genes encoding regulatory functions, we performed RACE-PCR and transcriptional fusion assays on the *mcsA-mcsB-clpC* operon and *hrcA*; qRT-PCR assays were performed to confirm the following: (i) σ^L-dependent transcription of *sigC*, (ii) σ^B-dependent transcription of *sigH*, (iii) σ^B- and σ^H-dependent transcription of *hfq*, (iv) σ^H-dependent transcription of *groES*, and (v) σ^B-dependent upregulation and HrcA-dependent repression of *cggR* (see Table S3 to S6 and S10 in the supplemental material).

For *mcsA*, in addition to the putative σ^B-dependent promoter identified by HMM at 309 nucleotides (nt) upstream of the predicted open reading frame, RACE-PCR with RNA from log-phase 10403S cells that had been exposed to 0.3 M NaCl for 10 min identified a second σ^B-dependent promoter 10 nt upstream of the *mcsA* transcriptional start site (see Fig. S1B in the supplemental material). The *hrcA* transcript mapped to a predicted σ^A-dependent promoter (see Fig. S1B), suggesting the possibility of *hrcA* transcription from both σ^A- and σ^B-dependent promoters following exposure to 0.3 M NaCl for 10 min. Promoter fusion assays confirmed σ^B-dependent transcription of both P_{mcsA} and P_{hrcA} in late-stationary-phase cells. Specifically, GUS activities were significantly higher in 10403S P_{hrcA}-*gus* and P_{mcsA}-*gus* strains than in Δ*sigB* P_{hrcA}-*gus* and P_{mcsA}-*gus* strains, respectively (Fig. 2). GUS activities in log-phase P_{hrcA}-*gus* and P_{mcsA}-*gus* cells that had been exposed to 0.3 M NaCl for 2 h were uniformly very low, likely because the σ^B response appears to be most pronounced within minutes of stress exposure (68); hence, σ^B-dependent regulatory patterns were not discernible from these data. qRT-PCR confirmed both *hrcA* and *clpC* as σ^B dependent in log-phase cells exposed to 0.3 M NaCl for 10 min but not in stationary-phase cells (grown to an OD_{600} of 1, followed by an additional 3-h incubation) (see Table S10). Despite the microarray evidence supporting σ^B-dependent transcription of *sigH* (i.e., transcript levels for *sigH* were 1.5-fold higher in 10403S than in the Δ*sigB* strain after exposure to salt stress [$P = 0.02$]), using qRT-PCR, *sigH* transcript levels were not statistically different between the 10403S and Δ*sigB* strains following exposure of log-phase cells to 0.3 M NaCl for 10 min. Similarly, qRT-PCR results did not verify σ^L-dependent expression of *sigC* during stationary phase using the 10403S and Δ*sigL* strains. Overall, our qRT-PCR data were able to verify a key role for σ^B in *L. monocytogenes* at least under the conditions tested, since we were able to confirm that transcription of a variety of other regulators is dependent on σ^B, including σ^B-dependent transcription of the following: (i) *hrcA*, (ii) *mcsA* and *clpC*, which are located in the same operon and contribute to posttranslational regulation of CtsR, and (iii) *hfq*, which encodes the RNA-binding protein Hfq, which appears to contribute to regulatory functions by interacting with numerous small regulatory RNAs (9).

Acid and heat stress resistance of strains with null mutations in genes encoding transcriptional regulators. Assessment of survival following a 1-h exposure to pH 2.5 shows, consistent with previous reports (8, 14, 15, 70), that survival of the Δ*sigB* strain (3.30 ± 0.53 log reduction) was significantly lower than that of 10403S (1.92 ± 0.52 log reduction). Survival of the Δ*sigH* (0.70 ± 0.86 log reduction) and Δ*sigL* (0.66 ± 0.22 log reduction) strains was significantly higher than that of the parent strain, while σ^C, PrfA, CtsR, and HrcA did not significantly contribute to acid stress survival under the conditions used in this assay (Table 5; see also Fig. S3 in the supplemental material).

CtsR was reported previously to contribute to the heat stress response in *L. monocytogenes* by repressing transcription of genes contributing to heat resistance with repression relieved in response to heat shock (30, 32, 33, 53). After exposure to heat (55°C for 1 h), survival of the Δ*ctsR* strain (2.79 ± 0.17 log reduction) was significantly higher than that of 10403S (4.63 ± 0.15 log reduction) (Table 5), consistent with findings of previous studies (30, 53) and the known role of CtsR as a transcriptional repressor of heat shock genes (e.g., the *clpB* operon [*clpB*-lmo2205 genes] and *clpP*). Other regulators, however, appeared to have minimal contributions to heat resistance since the log reductions for the remaining mutant strains (also after exposure to 55°C for 1 h) were similar to those for 10403S (Table 5; see also Fig. S4 in the supplemental material).

Characterization of virulence-associated phenotypes. (i) Invasion assay. Except for the reduced invasion efficiency of the Δ*sigB* strain, none of the other six strains with null mutations in transcriptional regulators showed differences in invasion efficiency for Caco-2 human intestinal epithelial cells from that of the isogenic parent strain when using stationary-phase bacteria for inoculation (Table 5; see also Fig. S5 in the supplemental material). The reduced invasion efficiency of the Δ*sigB* strain, which has been reported previously (20, 37, 38), is consistent with the known role for σ^B in regulating transcription of *inlA*, which is important for *L. monocytogenes* attachment and invasion in intestinal epithelium (37, 38). The observation that early-stationary-phase Δ*prfA* cells showed no significant reduction in invasion capacity compared to the parent strain suggests a limited role for PrfA in invasion of Caco-2 cells by stationary-phase bacteria (consistent with previous studies [37, 38]), despite the fact that transcription of the *inlAB* locus is regulated by both σ^B and PrfA and that PrfA has been shown to play a role in invasion in bacteria grown under other conditions (e.g., log phase) (38).

TABLE 5. Phenotypic characteristics of regulatory mutant strains[a]

Strain or mutation	Log reduction after 1 h of exposure to pH 2.5	Log reduction after 1 h of exposure to 55°C	Invasion efficiency in Caco-2 cells[c]	Intracellular growth in activated J774 cells at 7.5 h after inoculation[d]	*L. monocytogenes* recovery (log CFU/g) in inoculated guinea pigs at 72 h p.i. from[b]:				*L. monocytogenes* recovery (log CFU/g) from feces of infected guinea pigs at[b]:			Guinea pig wt at 72 h p.i. (% of initial wt)[b]
					Liver	Spleen	Mesenteric lymph nodes	Small intestine	24 h p.i.	48 h p.i.	72 h p.i.	
10403S	−1.92	−4.63	−2.90	1.75	3.64	3.57	5.00	4.80	7.38	5.85	5.52	91.47
Δ*sigH*	**−0.70†**	−4.19	−2.27	1.90	2.77	2.83	5.03	4.80	7.44	4.86	4.50	98.19
Δ*sigL*	**−0.66†**	−4.52	−2.76	1.58	3.07	3.04	4.95	4.77	7.16	**4.16***	4.95	97.27
Δ*sigC*	−1.38	−4.65	−2.41	1.76	2.84	2.81	4.94	4.63	7.03	**3.04***	4.74	103.69
Δ*sigB*[e]	**−3.30***	−5.28	**−4.06***	1.71	▼[e]	▼[e]	▼[e]	▼[e]	NS_{WT}[e]	▼[e]	▼[e]	NS_{UN}[e]
Δ*prfA*[e]	−1.16	−5.05	−2.72	**−0.21***	▼[e]	▼[e]	▼[e]	▼[e]	NS_{WT}[e]	▼[e]	▼[e]	NS_{UN}[e]
Δ*ctsR*	−1.19	**−2.79†**	−3.15	1.76	**1.91***	**2.05***	**3.91***	**3.43***	5.98	**2.14***	**1.98***	**107.74†**
Δ*hrcA*	−1.58	−4.53	−2.57	1.72	2.45	3.12	5.22	4.36	5.84	4.64	5.36	97.12

[a] Full data sets are available in Fig. S2 to S8 in the supplemental material. Values marked by "*" or "†" (and bolded) are significantly ($P < 0.05$) lower or higher, respectively, than values for the parent strain, 10403S (shown in first row).
[b] p.i., postinoculation.
[c] Invasion efficiency was calculated as log (CFU/ml recovered) − log (CFU/ml inoculated).
[d] Intracellular growth was calculated as log (CFU/ml recovered at $T = 7.5$) − log (CFU/ml inoculated at $T = 1.5$).
[e] Guinea pig infection data reported in this table for the Δ*sigB* and Δ*prfA* strains were previously reported by Garner et al. (20) and Oliver et al. (55); for these data, "▼" represents a significantly reduced count in comparison to that for 10403S; NS_{WT}, no significant differences in *L. monocytogenes* counts in fecal samples from those for guinea pigs infected with 10403S (wild type); NS_{UN}, no significant differences in percent weight from that of uninfected guinea pigs.

(ii) Intracellular growth assay. *L. monocytogenes* is a facultative intracellular pathogen; therefore, potential roles were investigated for each of the regulators in multiplication and spread in the intracellular microenvironment using intracellular growth assays with LPS-activated J774 macrophages (Table 5; see also Fig. S6 in the supplemental material). As shown previously (17, 23), the Δ*prfA* strain showed a limited ability to multiply inside the cell. The Δ*sigB* strains showed intracellular growth patterns similar to those of 10403S, indicating that σ^B is not essential for intracellular growth. Likewise, none of the remaining mutants exhibited growth impairment within the macrophages. We conclude that intracellular growth in J774 macrophages is not critically dependent on any of these transcriptional regulators other than PrfA, at least under the conditions used in this assay.

(iii) Guinea pig model. To gain a more comprehensive understanding of the contributions of transcriptional regulators and specifically of σ^H, σ^L, σ^C, CtsR, and HrcA to virulence, *L. monocytogenes* mutant strains lacking these regulators were tested for virulence in the guinea pig model. The Δ*ctsR* strain showed significantly lower recovery (in log CFU/g organ) of *L. monocytogenes* from all harvested organs, i.e., liver, mesenteric lymph nodes, spleen, and the distal section of the small intestine (Table 5; see also Fig. S7 in the supplemental material), than the parent strain, suggesting attenuated virulence. In addition, animals infected with the Δ*ctsR* strain showed the lowest *L. monocytogenes* levels in their feces (Table 5; see also Fig. S8) and had the greatest positive percent weight change (excluding the control) (Table 5; see also Fig. S9) during the experimental period. With the exception of reduced bacterial numbers for the Δ*sigC* and Δ*sigL* strains in fecal matter at 48 h postinfection, the other strains lacking transcriptional regulators showed no detectable virulence differences from 10403S. In conjunction with previous findings (20), we have thus established that, in addition to σ^B and PrfA, CtsR contributes to *L. monocytogenes* intragastric infection in the guinea pig model.

DISCUSSION

A comprehensive analysis of regulatory networks involving *L. monocytogenes* σ^B, σ^H, σ^L, σ^C, PrfA, CtsR, and HrcA revealed the following: (i) considerable overlap among regulons, including 188 coregulated genes with the majority coregulated by σ^B, suggesting a central role for σ^B in transcriptional regulation in *L. monocytogenes*, and (ii) a number of cross-connections between transcriptional regulators, including σ^B-dependent regulation of *prfA*, *hrcA*, *mcsA* and *clpC*, and *hfq*, further confirming a key role for σ^B in *L. monocytogenes* transcriptional networks. Phenotypic evaluation of selected stress response and virulence phenotypes in null mutants lacking key transcriptional regulators showed that deletions of σ^B, PrfA, and CtsR show clear phenotypic consequences in assays assessing virulence-associated characteristics (including guinea pig infections) and in some but not all stress response assays. Importantly, while the Δ*ctsR* strain was previously reported to have enhanced survival under heat stress (30), results from the present study indicate a role for CtsR in virulence, since we observed significantly reduced bacterial numbers of the Δ*ctsR* strain in infected guinea pigs.

Transcription factors show considerable overlap in *L. monocytogenes*. A key finding from this study is the extensive regulon overlaps for the various transcriptional regulators under defined conditions. σ^B clearly plays an important role in overall *L. monocytogenes* transcriptional regulation, since the largest regulon overlaps occur between σ^B and other regulators (in particular, σ^H and σ^L). Interrelationships between *L. monocytogenes* σ^B and the negative regulators CtsR and HrcA and between σ^B and the positive regulator PrfA have been reported previously (5, 29, 30, 50, 56, 69). In terms of contribution to biological functions, genes coregulated by σ^B and PrfA (e.g., *inlA* [34, 38, 49]) have previously been shown to be critical for virulence (18, 42), even though contributions of σ^B and PrfA to *inlA* transcription may be apparent only under specific growth conditions (38, 48). Our data here also indicate

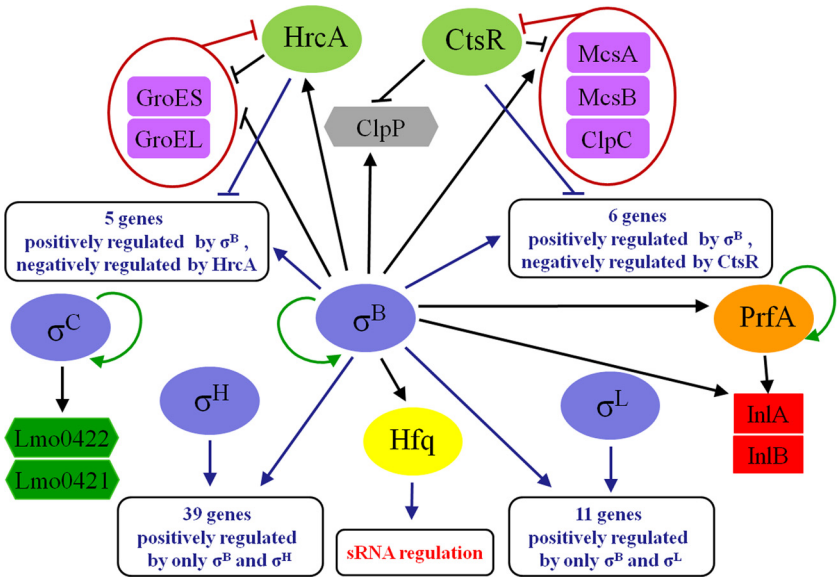

FIG. 3. Partial transcriptional interaction network based on microarray data and TaqMan and RACE results for the alternative sigma factors σ^B, σ^C, σ^H, and σ^L (blue ellipses), the virulence gene regulator PrfA (orange ellipse), and the negative regulators CtsR and HrcA (green ellipses). Color-coded shapes were used to identify transcriptional regulators (yellow ellipse), regulators of transcriptional regulators (purple rounded rectangles), virulence proteins (red rectangles), stress response proteins (gray hexagons), and σ^C-dependent genes (green hexagons). Genes arranged in vertical columns represent operons. Black target arrows ($\downarrow$) originating from a given regulator indicate positive regulation; black target stops ($\perp$) indicate negative regulation by a given regulator. Blue arrows or target stops target groups of genes coregulated by more than one regulator (blue font); in addition, a blue arrow indicates that Hfq regulates small RNAs ("sRNA regulation"). Green loops indicate autoregulation. The red target stop directed at HrcA indicates posttranscriptional regulation of HrcA by GroES and GroEL, as shown in *B. subtilis* (51); the red target stop directed at CtsR indicates posttranscriptional regulation of CtsR by McsA, McsB, and ClpC, as shown in *B. subtilis* (39–41).

coregulation of motility-related functions by σ^B and a number of other regulators; negative regulation of expression of MogR, a repressor of flagellar biosynthesis, has recently been shown to be due to a σ^B-dependent long 5′ untranslated region (5′ UTR) RNA (69). Since motility and motility genes have been shown to contribute to *L. monocytogenes* virulence (25, 57), these data illustrate the complex contributions to virulence of σ^B and regulatory networks involving σ^B. In addition to virulence-associated functions, our data also suggest that coregulation by σ^B and other regulators is important for other functions, such as energy metabolism (e.g., biosynthesis/degradation of polysaccharides and glycolysis/gluconeogenesis), as illustrated by the genes classified in SOTA clusters 9, 10, and 13, which are all upregulated by σ^B and σ^H in addition to regulatory contributions by other regulators. Therefore, in addition to the fact that the σ^B regulon controls the largest number of genes relative to other regulators studied thus far, our findings confirm that σ^B plays a central role in modulating gene expression in *L. monocytogenes*. σ^B is an important cocontributor to transcriptional regulation in other Gram-positive microbes, as well. For example, in *B. subtilis*, dual regulation of a gene set by σ^B and σ^H is important when bacteria encounter amino acid starvation (11, 12).

Regulatory circuits exist among transcriptional regulators in *L. monocytogenes*. In addition to identification of regulon overlaps, which imply shared biological functions for regulatory proteins, our data also indicate that several transcriptional regulators control other regulators (Fig. 3). We propose that these relationships may be important for enabling cascades or amplifications of signals, and hence responses, under specific environmental conditions. One example of such a regulatory network is illustrated by σ^B-dependent regulation of genes encoding other regulators and chaperones, such as *hrcA*, *mcsA*, *groES*, and *hfq*, in addition to contributions of σ^B to regulating transcription of *prfA* (52, 60, 64). Regulatory control of expression of these genes by σ^B provides additional support for the hypotheses that σ^B is the following: (i) a core modulator of global gene expression in *L. monocytogenes*, including through contributions to regulating expression of other subregulons (e.g., those of HrcA and CtsR), and (ii) a cross talk modulator of transcriptional, posttranscriptional, and translational processes in *L. monocytogenes*. Specific examples of these processes include the following: (i) σ^B-dependent contributions to transcription of *ctsR* and *prfA* (representing transcriptional cross talk), (ii) σ^B-dependent contributions to transcription of *hfq*, which encodes a protein that contributes to regulatory functions by interacting with small regulatory RNAs (9, 10, 54) (representing posttranslational cross talk), and (iii) σ^B-dependent contributions to transcription of *mcsA* and *clpC*, which encode molecular chaperones that determine protein fate and contribute to posttranslational regulation of CtsR (representing posttranslational cross talk). Similar networks have been found in *B. subtilis*, in which σ^B regulates MgsR, a paralog of the oxidative stress regulator Spx, which subsequently regulates a "subregulon" of *B. subtilis* σ^B (62). For all regulons characterized in this study, we also observed negative regulation of genes by transcription factors with assumed positive roles (i.e., all alternative sigma factors and PrfA) and positive regulation of genes by negative regulators (i.e., CtsR and HrcA). These observations suggest indirect regulation, e.g., to

compensate for differential regulation of genes directly regulated by a given regulator, or could be a consequence of regulatory cascades (e.g., through regulation of genes encoding other regulators). Further experiments are needed, though, to confirm these transcriptional patterns and to understand the actual mechanisms of indirect regulation.

Phenotypic characterization suggests role redundancies among *L. monocytogenes* regulators. While an *L. monocytogenes* EGD-e *sigL* mutant was previously reported to show impaired growth relative to the wild-type strain at low temperature, in the presence of salt, and under lactic acid stress (59) and while an *L. monocytogenes sigH* mutant has shown reduced growth in minimal medium and in alkaline conditions compared to the wild-type strain (61), the absence of phenotypic consequences from in-frame deletions in *sigH*, *sigL*, and *sigC*, under the conditions used here, is consistent with the observation of overlapping regulons for these proteins, which implies functional redundancies among these regulators. Redundancy has been observed among ECF sigma factors in *B. subtilis*. Specifically, loss of multiple ECF sigma factors did not result in observable phenotypes (2). These observations suggest a compensation phenomenon, which is consistent with previous observations that bacteria can tolerate and adapt to changes in genome components, loss of major regulators, and changes in regulatory circuits without obvious phenotypic variations (31). Previous data also suggest that loss of one alternative sigma factor shifts the balance of sigma factor and RNA polymerase pools, typically in favor of a housekeeping sigma factor that is already abundant, resulting in compensation of gene expression by σ^A, with little to no changes in phenotype when two or more sigma factors share an upstream regulatory region (e.g., overlapping recognition sites) (24, 43, 63). While microarray data without additional confirmation can provide relevant data on genome-wide analyses of regulon and transcriptional patterns, which rely on overall data patterns (rather than information on transcript patterns for a single individual gene) (46), additional validation of coregulation for individual genes and identification of promoter sites will be needed to further characterize mechanisms contributing to the coregulation patterns observed here.

Conclusions. Initial identification and characterization of the σ^H and σ^C regulons in *L. monocytogenes* along with comprehensive bioinformatics analysis of the regulons for 7 key transcriptional regulators (σ^H, σ^L, σ^C, σ^B, PrfA, CtsR, and HrcA) support a transcriptional regulatory network in *L. monocytogenes*, with σ^B as a central regulator (Fig. 3) and a particularly prominent overlap between the σ^B and σ^H regulons. The overlapping regulons identified here suggest the existence of complex mechanisms, likely to be both compensatory and synergistic, to enable fine-tuning of gene expression in response to changing environmental conditions. While a number of instances of cross-regulation of one transcriptional regulator by another transcriptional regulator (e.g., σ^B-dependent transcription of PrfA [52, 60, 64] and σ^B-dependent transcription of MogR [69]) have been identified so far, coregulation of individual genes (e.g., σ^B- and HrcA-dependent expression of a heat shock response *groES-groEL* operon [29]) is also critical to these regulatory networks. Despite the fact that some initial components of the regulatory networks in *L. monocytogenes* have been identified so far (29, 30, 56), it is also clear that we are only beginning to understand the complexities of transcriptional regulation in *L. monocytogenes*. The regulon overlaps, as well as the observation that *sigH*, *sigC*, and *sigL* null mutations showed limited phenotypic effects, suggest that some of these regulators may be important predominantly for specific biological functions or under certain environmental conditions (as shown for a number of other bacteria, e.g., FliA in *Salmonella enterica* and RpoE in *Escherichia coli* [35]) and that contributions of these regulators may be apparent only in mutant strains that carry only a single sigma factor (thus eliminating redundancies and compensation), representing an approach that has been used in *B. subtilis* (44). Future phenotypic and gene expression experiments using *L. monocytogenes* grown under different environmental conditions (e.g., different temperatures) and strains that eliminate regulatory redundancies (e.g., strains with deletions of multiple or all alternative sigma factors) will thus be needed to further advance our knowledge of regulatory networks in *L. monocytogenes.* Furthermore, studies in different *L. monocytogenes* strains (as recently described for σ^B [55]) will also be necessary to better understand regulatory networks since it is increasingly clear that subtypes and strains within a bacterial species can differ considerably in gene regulation.

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